

Mechanism of Enteroviral Inactivation by Ozone

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The mechanism of enteroviral inactivation by ozone was investigated with poliovirus 1 (Mahoney) as the model virus. Ozone was observed to alter two of the four polypeptide chains present in the viral protein coat of poliovirus 1. However, the alteration of the protein coat did not significantly impair virus adsorption or alter the integrity of the virus particle. Damage to the viral RNA after exposure to ozone was demonstrated by velocity sedimentation analysis. It was concluded that the damage to the viral nucleic acid is the major cause of poliovirus 1 inactivation by ozone.

Ozone has been known for years to be a strong disinfecting agent. Although the ability of ozone to inactivate enteric viruses has been documented in the literature by many researchers, little attention has been directed toward the determination of the mechanism of ozone inactivation of viruses. Information regarding the mechanism of inactivation would be useful in developing mathematical models to predict the performance of the ozone disinfection process as applied to the inactivation of viruses in water and wastewater treatment.

Enteroviruses consist of a single-stranded ribonucleic acid (RNA) of 2×10^6 daltons enclosed in a protein coat consisting of 60 capsomeres. The protein coat is about 6 nm thick with a total molecular weight of 5.7×10^6 . Each capsomere has a molecular weight of approximately 95,000 and is made up of four polypeptide chains having molecular weights of 35,000, 28,000, 24,000, and 8,000 (7).

Poliovirus infection is initiated by adsorption of virus onto the host cells. After the uncoating of the protein coat, viral RNA is released into the cell cytoplasm to initiate the synthesis of the proteins and the replication of RNA. After an eclipse phase, as reported by Martin and Work (11), viral RNA, protein, and then the mature viruses appear. It is evident that any compound, to be effective as a virucidal agent, should be capable of causing physical disruption of the virion or reacting with the protein coat or the viral RNA or both such that one or more of the steps of viral replication is blocked.

The objective of this research was to elucidate the mechanism of enteroviral inactivation by ozone by identifying the major reaction(s) and the lethal site(s) involved in the inactivation process.

MATERIALS AND METHODS

Glassware. All glassware was thoroughly cleaned to ensure that it was ozone demand free. It was cleaned with a dichromate-sulfuric acid mixture, rinsed in tap water followed by distilled water, and finally soaked in a strong ozone solution before drying at 180°C for at least 6 h to ensure the elimination of ozone.

Virus. Poliovirus type 1 (Mahoney), originally obtained from Gerald Berg, Environmental Protection Agency, Cincinnati, Ohio, was used as the model enteric virus.

Cell line. An African green (*Cercopithecus aethiops*) monkey kidney cell line known as BGM (buffalo green monkey) was used for virus propagation and titration by plaque assay. BGM cells were grown in medium 199 (Grand Island Biological Co., Grand Island, N.Y.) supplemented with 10% fetal calf serum (Grand Island Biological Co.), 0.2% sodium bicarbonate, and 1% antimycotic antibiotic (Grand Island Biological Co.). The cell line was maintained by continuous passage at a concentration of 1.2×10^6 cells per ml.

Ozone demand-free water. Distilled deionized water was ozonated for 15 min to oxidize any trace organic matter and then boiled for 30 min to dissipate residual ozone. The treated water was then stored under ultraviolet light until used.

Buffers. Buffers to achieve the two pH values used in this study were prepared as follows: (i) (pH 7.2) 1.967 g of sodium dihydrogen phosphate ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$) and 5.074 g of anhydrous disodium hydrogen phosphate (Na_2HPO_4) were dissolved in 5 liters of ozone demand-free water; (ii) (pH 4.3) 2.6 ml of concentrated acetic acid (CH_3COOH) and 0.645 g of anhydrous sodium acetate (CH_3COONa) were dissolved in 5 liters of ozone demand-free water.

Phosphate-buffered saline solution. Concentrated (10 times) phosphate-buffered saline solution was prepared by the procedure described by Rovozzo and Burke (15).

Preparation and purification of viruses. Flasks containing a confluent monolayer of BGM cells were washed twice with serum-free growth medium and inoculated with a stock virus suspension. Virus adsorption was allowed to occur for 1 h in an atmosphere of 5% CO_2 at 37°C. After 24 to 36 h of incubation and

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before further purification, each flask was frozen at -70°C .

The crude suspension containing virus-infected cells was purified by a technique similar to that described by Scarpino et al. (18). In this procedure, the crude suspension was frozen at -70°C and thawed three times, followed by centrifugation at 10,000 rpm for 1 h to remove the cell debris. The clarified supernatant was frozen once again at -70°C , thawed, and then centrifuged at 40,000 rpm for 5 h. The pelleted virus was suspended in buffer appropriate for the intended experiments. The total organic carbon of such a virus preparation, having a titer of 10^6 plaque-forming units per ml, was determined by an ultralow total organic carbon analyzer (Dohrman, Envirotech, Santa Clara, Calif.) to be on the order of $270\text{ }\mu\text{g/liter}$. This level of total organic carbon was found to exert negligible ozone demand in batch disinfection system as used in this study (16).

Labeled virus was prepared by growth in medium containing either ^{14}C -amino acid mixture at $5\text{ }\mu\text{Ci/ml}$ (Amersham Corp., Arlington Heights, Ill.) or ^3H -uridine at $10\text{ }\mu\text{Ci/ml}$ for 24 h and purified as described above.

Virus assay. Virus titers were determined by plaque assay, with confluent BGM monolayers in tissue culture plates (60 by 15 mm; Falcon Division, Bioquest, Oxnard, Calif.). Serial dilutions of the virus suspension were prepared with Hanks balanced salt solution (Grand Island Biological Co.), containing 2% fetal calf serum, 0.5% thiosulfate, 1% antimycotic antibiotic, and 0.5% sodium bicarbonate. After inoculation and adsorption at 37°C under 5% CO_2 for 1 to 2 h, the cells were overlaid with 5 ml of agar medium, consisting of medium 199 plus 2% fetal calf serum, 1% magnesium chloride, 1% antimycotic antibiotic, 0.5% sodium bicarbonate, and 0.9% agar, and reincubated at 37°C until the appearance of plaques. At this time, the monolayer was fixed with 70% ethanol, formaldehyde, and acetic acid in a volume ratio of 20:1:1. The monolayer was then stained with a 1% solution of crystal violet, and plaques were counted.

Gel electrophoresis. Electrophoretic analysis of the poliovirus capsid polypeptides labeled with ^{14}C -amino acids was performed by the sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis method described by Vrijse and Boeye (20). Gel columns, 9 cm long, containing 12.5% polyacrylamide, 0.1% SDS, and 0.1 M phosphate buffer, were prepared in glass tubes having an inside diameter of 6 mm. The pH of the gel was 7.2. Each sample, consisting of 0.3 ml, was layered on gel, and electrophoresis was performed with a conventional gel electrophoresis cell for 24 h at 25 V with both electrode buffers at pH 7.2.

After electrophoresis, the gels were sliced using a Mickle gel slicer (Brinkman, Des Plaines, Ill.), and slices were transferred into glass vials, solubilized in 0.5 ml of hydrogen peroxide at 65°C for 24 h, and radioactivity was determined by scintillation spectrometry.

Extraction and analysis of viral RNA. Viral RNA was extracted from purified poliovirus by the method developed by Mandel (10), who showed that the efficiency of RNA extraction after disruption of the capsid by SDS is greatest at acidic pH values ranging from 3.5 to 4.4. In view of this, extraction and

analysis of viral RNA was performed at pH 4.3. Acetic acid buffer at pH 4.3 (2.4 ml) and 10% SDS solution (0.3 ml) were added to 0.3 ml of viral suspension. The mixture was held at room temperature for 1 h and then centrifuged on a 5 to 20% sucrose density gradient at 35,000 rpm for 210 min at 20°C .

Viral attachment experiment. Virus samples labeled with either ^3H -uridine or ^{14}C -amino acids were inoculated on BGM cell monolayers and incubated for 60 to 90 min in an atmosphere of 5% CO_2 at 37°C . After incubation, the cell monolayers were washed three times with phosphate-buffered saline solution to remove unattached viruses. The cell layer was then removed with a rubber policeman and collected in a centrifuge tube. The cell suspension was pelleted by centrifugation at 3,000 rpm for 20 min. The cell pellet, which did not contain any unattached viruses, was then suspended, and the associated radioactivity was determined.

Ozonation procedure. The experiments carried out for elucidation of the mechanism of enteroviral inactivation by ozone were performed in a batch system using glass tubes at room temperature. The procedures of ozone generation and preparation of aqueous solution of ozone have been reported earlier (16). An aqueous solution of ozone of known concentration was added to the tube containing radioactive viruses and was manually mixed for a predetermined contact time. An appropriate volume of 0.5 M sodium thiosulfate solution was then added to the tubes to neutralize any residual ozone. Samples were then analyzed as required for the particular study.

Survival of viruses after exposure to ozone is dependent on the residual ozone concentration. Kinetics of inactivation of poliovirus 1 using various residual ozone concentrations have been presented earlier (16). It was observed that a 0.21-mg/liter residual ozone concentration at pH 7.2 for 30 s resulted in approximately 99% inactivation of poliovirus 1. Similar studies by Katzenelson et al. (6) reported that between 0.1 to 0.2 mg/liter ozone residual, the percent survival decreased with increasing residual concentration. However, increasing the ozone residual from 0.2 to 1.5 mg/liter did not alter the percent survival. In view of the above findings, residual ozone concentrations were maintained in the range of 0.3 to 0.8 mg/liter in all experiments of this study to insure at least 99% inactivation of viruses.

The spectrophotometric method of ozone measurement developed by Schechter (19) was used to monitor the aqueous concentration of ozone.

RESULTS

Effect of ozone on structural integrity of the virus. To determine whether the overall integrity or the structure of a virus particle was significantly altered after exposure to a low concentration of ozone, a preparation of ^3H -uridine-labeled poliovirus 1 was exposed to 0.32-mg/liter residual ozone concentration for 30 s at room temperature. Untreated and ozone-treated labeled virus preparations were then centrifuged on 10 to 40% sucrose gradients for 2 h at 35,000 rpm at 5°C and then fractionated, and radioac-

tivity was determined. As shown in Fig. 1, both ozone-treated and untreated virus preparations yielded a single peak of radioactivity, indicating an essentially homogeneous preparation of intact virus particles. The sedimentation coefficient for the peak was calculated to be 158S by the approximate method of Griffith (5); this value agrees with the range of 153 to 160S reported for single poliovirus particles by Ruckert (17).

If [^3H]uridine-labeled viruses were so severely damaged by ozone as to cause disintegration, then the nucleic acid would have leaked out, in which case nucleic acids having a much lower molecular weight would have sedimented as a band at a different location. These results suggest that the physical integrity of the virus did not change significantly as a result of exposure to ozone, i.e., the majority of the viral population remained as single intact virions.

Effect of ozone on viral protein. Although there was no evidence that the viral protein coat was dissociated under these conditions (Fig. 1), partial damage or some change in one or more polypeptide chains could not be ruled out. To determine whether any change in the viral cap-

sid did occur due to ozonation, viruses labeled with ^{14}C -amino acid were exposed to 0.7-mg/liter residual ozone concentration for 60 s and then analyzed by SDS-polyacrylamide gel electrophoresis. Labeled viruses, not exposed to ozone, were analyzed in parallel for comparison. The electrophoretic profile of the unexposed viruses (Fig. 2A) revealed three large peaks corresponding to polypeptide chains VP1, VP2, and VP3, and a small peak, VP4. The structural polypeptides of unexposed viruses identified in this experiment are in agreement with those reported by Maizel et al. (9). Comparison of this protein profile with that of ozone-exposed virus (Fig. 2B) suggests possible damage to polypeptide chains VP1 and VP2. In addition, there was the appearance of a new peak with fraction no. 19, suggesting the possible breakdown of the large-molecular-weight polypeptide chains. Polypeptide chain VP4 appears to be unaffected by ozone treatment. In the case of enteroviruses, it has been shown that VP4 is responsible for virus attachment (1, 3, 4, 8). The protein profiles shown here and the adsorption studies discussed below indicate that inactivation of enteroviruses by a low dosage of ozone cannot be attributed to the damage of the viral capsid polypeptide affecting attachment or penetration or both.

Effect of ozone on viral attachment-penetration. Inactivation of poliovirus 1 by ozone was found to be associated with a change in the polypeptide structure of the protein coat. Although such a change did not cause disintegration of the viral particles, the possibility of affecting the attachment of the virus particles on the host cells could not be ruled out. After attachment, viruses penetrate the host cells by a process which has not been completely identified to date. Because of this difficulty, in the current study these two processes, attachment and penetration, are treated as one process.

The polypeptide mapping, as discussed in the previous section, indicated that the polypeptide chain VP4 remained unchanged as a result of ozonation. In view of this observation, two sets of experiments were performed to determine whether ozone-exposed poliovirus showing a change in VP1 and VP2 but no change in VP4 was affected with respect to its ability to attach to host cells.

In the first set of experiments, different samples of the same preparation of poliovirus 1 labeled with [^3H]uridine were exposed to various residual ozone concentrations for 30 s at pH 7.2 and inoculated onto cell monolayers. After incubation for attachment, cell-associated radioactivity was measured; the results are presented in Fig. 3. Percent penetration-attachment was calculated as the ratio of cell-associated radio-

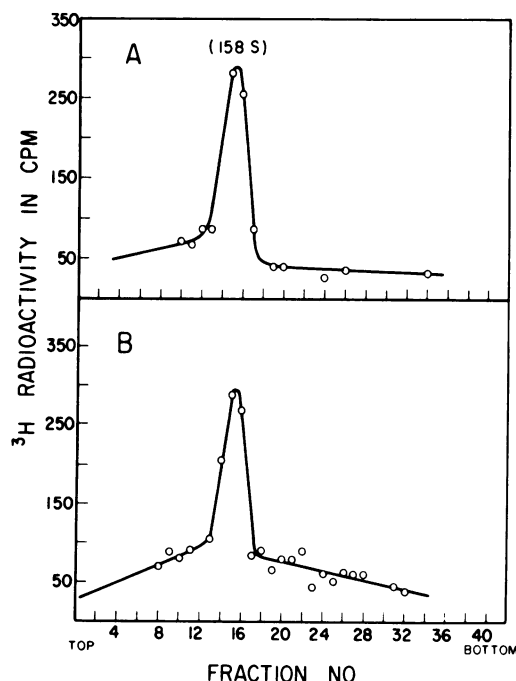


FIG. 1. Centrifugation analysis of [^3H]uridine-labeled poliovirus 1 on 10 to 40% sucrose density gradients for 2 h at 35,000 rpm and 5°C for (A) control virus suspension; and (B) viruses exposed to 0.32-mg/liter residual ozone concentration for 30 s.

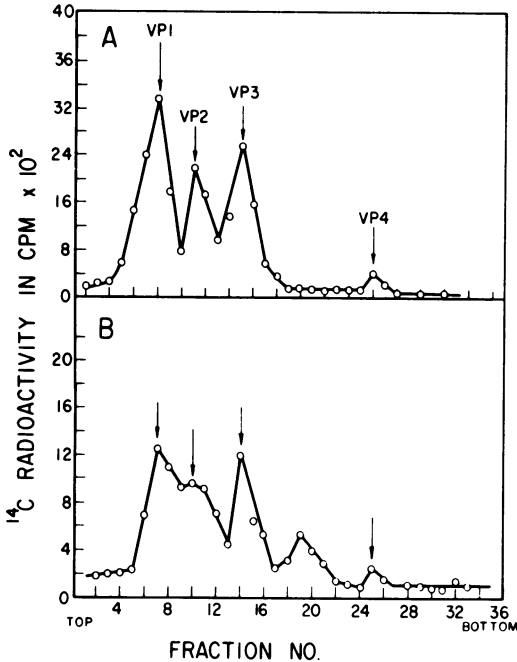


FIG. 2. SDS-polyacrylamide gel electrophoresis pattern of poliovirus 1 proteins from (A) control virus suspension and (B) poliovirus 1 exposed to 0.7-mg/liter residual ozone concentration for 60 s. Polioviruses were labeled with ^{14}C -amino acid mixture, and gel electrophoresis was performed by the method described in the text.

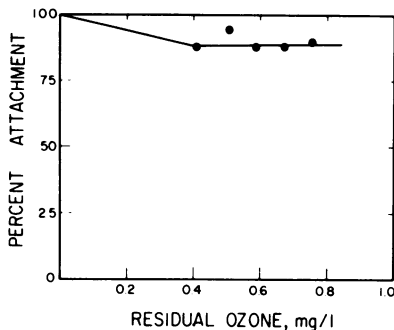


FIG. 3. Effect on poliovirus 1 penetration-attachment to BGM host cell after exposure to various ozone residual concentrations for 30 s at pH 7.2. Viruses were labeled with $[^3\text{H}]$ uridine.

activity imparted by the labeled virus to that of the labeled control virus suspension. The data presented in Fig. 3 indicate that the reduction in percent virus penetration-attachment was not markedly affected by the exposure of the viruses to ozone residual concentrations up to 0.8 mg/liter.

The second set of experiments was performed

with 0.51-mg/liter residual ozone concentration at pH 7.2; ^{14}C -amino acid-labeled poliovirus was exposed to this residual concentration of ozone for different contact times. The results of this set of experiments are presented in Fig. 4. The reduction in percent penetration-attachment obtained in this set of experiments was higher than that of the previous set with $[^3\text{H}]$ uridine-labeled virus. This increased reduction may be due to the loss of radioactivity from the polypeptide chains VP1 and VP2, which do not take part in the attachment process. Approximately 1 log difference was observed between the survival curve and penetration curve (Fig. 4), indicating that the impairment of viral coat protein may not be the major cause of inactivation.

Effect of ozone of viral RNA. To determine whether viral RNA is damaged by exposure to ozone, $[^3\text{H}]$ uridine-labeled poliovirus was treated with 0.31-mg/liter residual ozone concentration at pH 4.3 for 45 s. The RNA from the control and ozone-treated samples were then extracted and centrifuged on a 5 to 20% sucrose density gradient at 35,000 rpm for 210 min. Fractions collected from the density gradient analysis were then assayed to determine the radioactivity associated with each fraction. The results of these experiments are presented in Fig. 5A and 5B.

The sedimentation profile of the RNA extracted from the control virus (Fig. 5A) indicates

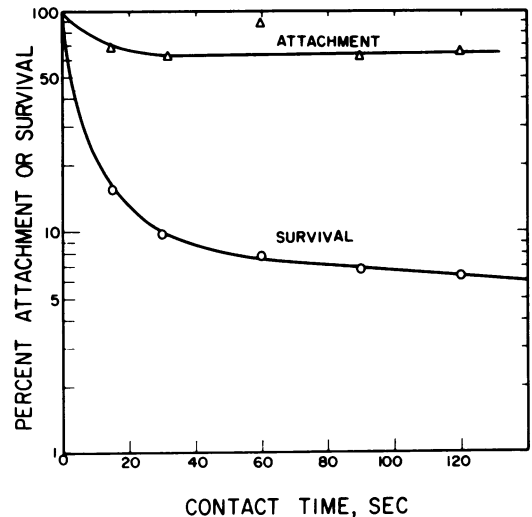


FIG. 4. Effect on poliovirus 1 penetration-attachment to BGM host cell and survival in a batch system using 0.51-mg/liter ozone residual concentration at 20°C, pH 7.2. Viruses were labeled with ^{14}C -amino acid mixture. Percent survival was calculated as the ratio of titers of the exposed viruses to that of control virus suspension.

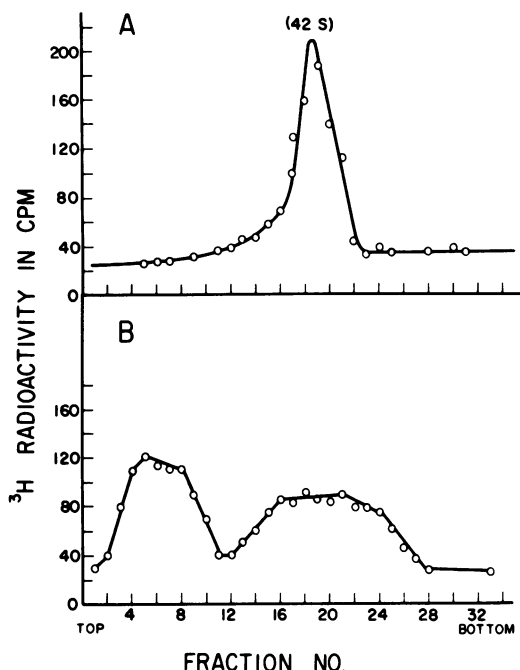


FIG. 5. Centrifugation analysis of [^3H]uridine-labeled poliovirus 1 RNA on a 5 to 20% sucrose density gradient at 35,000 rpm for 210 min. (A) RNA extracted from control virus suspension; (B) RNA extracted from ozonated poliovirus 1 suspension exposed to 0.3-mg/liter residual for 30 s at pH 4.3.

a single peak. The sedimentation coefficient of the RNA at the peak was calculated to be 42S by the approximate method of Griffith (5). The sedimentation coefficient of the RNA (42S) corresponds well with the reported sedimentation coefficient of poliovirus RNA (37S) in the literature (17), suggesting that the peak obtained in the sedimentation profile of these experiments represented the single-stranded RNA of the poliovirus. The sedimentation profile of RNA of the ozone-treated viruses (Fig. 5B) indicates that the viral RNA did not sediment in a single band; rather, two broad bands were observed. This observation implies that the viral RNA was damaged due to ozonation, possibly fragmented into a number of short chains.

DISCUSSION

Inactivation of viruses is defined as their inability to replicate within host cells. Considering the structure of the enteric viruses, it is reasonable to assume that ozone causes physical disruption of the particle or reacts with either the viral coat protein or the nucleic acid or both so as to affect attachment or intracellular step(s) in virus replication. Thus, barring physical disruption, damage to an enteric virus particle could

affect: (i) extracellular reactions taking place between the host cells and the virus particles leading to attachment and subsequent penetration of viruses into the host cells; or (ii) intracellular biosynthesis taking place after viral genome penetration into the host cell; or both.

Extracellular reactions are initiated when the invading virus particle is adsorbed to the surface of a susceptible host cell. This probably involves an interaction between the viral capsid protein and the specific components of the host cell surface. Rekosh (13), in a review article on molecular biology of viruses, suggested that the small virus polypeptide VP4, present within the virion, plays an important role in the adsorption process. Crowell and Philipson (4) and Longer-Holm and Kornat (8) demonstrated that both coxsackievirus and rhinovirus, which lack VP4, can be eluted from the host cells to which they had attached. Also, Cords et al. (3) and Breindl and Koch (1) have reported that a loss in host cell infectivity is associated with a loss of polypeptide chain VP4 with both poliovirus and coxsackievirus.

In studies designed to determine the mechanism of poliovirus inactivation by ozone, Riesser et al. (14) reported that upon reaction with ozone, the capsid of the virus particle is damaged. They concluded that this, in turn, affects the attachment of the virus to a susceptible host cell. However, their results indicated that although there was little change in percent penetration during the first 2 min of exposure to ozone, there was a significant loss of viability, indicating that damage of the viral capsid might not be the major cause of viral inactivation by ozone.

Other research work relating to the effect of ozone on compounds like proteins and nucleic acids is worthy of mentioning at this point, as these are the two major constituents of a virus particle. The reaction of ozone with amino acids proteins has been reviewed by Mudd et al. (12). They reported that cysteine, methionine, tryptophan, tyrosine, histidine, cystine, and phenylalanine are oxidized by ozone, whereas other amino acids are unaffected by ozone. The effect of ozone on nucleic acids was studied by Christensen and Giese (2). Since absorbance at a wavelength of 260 nm is approximately proportional to the concentration of nucleic acids, they used this measurement to determine the effect of ozone on nucleic acid. After 15 s of exposure to ozone, the peak absorbance decreased to 50% of the control, and within 60 s absorbance at 260 nm was undetectable.

In this study, inactivation experiments were performed in batch systems using radiolabeled poliovirus 1 to investigate the mechanism of

inactivation by ozone. Exposure of viruses to ozone did not result in their dissociation into molecular subunits. Also, the viruses did not appear to form large aggregates after ozonation.

Comparison of the electrophoretic profiles of ozone-treated viruses with those of control viruses revealed that two polypeptide chains of the viral capsid, VP1 and VP2, were damaged due to ozonation. The smallest viral polypeptide chain of the capsid that has been reported to be involved in attachment (VP4) was not significantly altered by ozonation. Attachment of ozone-exposed viruses was observed to be less than that of the control viruses. However, the difference in percent attachment cannot account for the high percent inactivation that can be achieved for the same ozone dosage. Thus, it may be concluded that the ability of viruses to attach to host cells is not significantly affected as a result of exposure to a low residual concentration of ozone. This conclusion is consistent with the fact that polypeptide chain VP4, which has been reported to be associated with attachment, was not affected as a result of ozonation in this study.

By comparing the sedimentation profiles of nucleic acids of ozone-exposed viruses with those of control viruses, we observed that viral nucleic acid was damaged as a result of ozonation. It would appear that the major cause of viral inactivation, as determined with poliovirus 1, by ozone using a residual concentration less than 0.3 mg/liter and a contact time up to 2 min is damage of the RNA. This conclusion agrees with the results of the kinetic studies (16), which reported that the inactivation of viruses by ozone is rate limited by the diffusional step of ozone through the protein coat into the nucleic acid core.

ACKNOWLEDGMENTS

This research was partially supported by the funds received under an interagency agreement between the U.S. Army Medical Research and Development Command and the U.S. Environmental Protection Agency (DADA 17-72-C-2125, DAMD 17-75-C-5006).

We gratefully acknowledge the technical assistance provided by M. M. Soong, Department of Microbiology, University of Illinois, Urbana.

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